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Protein and Lipid Composition of *Colias philodice* and *C. eurytheme* Spermatophores and Their Changes Over Time (Pieridae)

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Abstract. During copulation male *Colias philodice* and *C. eurytheme* accessory gland secretions fill the appendix bursa and form a spermatophore in the female's bursa copulatrix. These secretions represent 6-7 percent of male body mass. Protein represents 10 and 23 percent of the dry mass of spermatophores and appendix bursa contents respectively. Total lipid in spermatophores was nearly equal to protein content. Hydrocarbons, nonhydrocarbons, and phospholipids represent 47, 41, and 12 percent, respectively, of total lipid. Hydrocarbons were composed of 25 components ranging from 19 to 38 carbon atoms. Ninety-three percent of total hydrocarbons were n-alkanes. Nearly equal amounts of cholesterol, diacylglycerols, triacylglycerols, and free fatty acids accounted for the nonhydrocarbon fraction.

Proteins and hydrocarbons leave the spermatophore, either intact or following degradation, more rapidly than the nonhydrocarbon and phospholipid fractions which stay within the spermatophore until the spermatophore is almost completely deflated.

Since other studies have demonstrated transfer of protein from male to female, and its subsequent use in egg production, the implications of presence of lipids in spermatophores are discussed.

Introduction

In many insects, a spermatophore is passed during copulation. The nonsperm portion of the spermatophore is composed of male accessory gland secretions that are absorbed by the female and possibly used for maintenance or egg production (Thornhill, 1976; Friedel & Gillott, 1977; Boggs & Gilbert, 1979; Boggs, 1981; Boggs & Watt, 1981). Oviposition is stimulated by these accessory secretions in Orthoptera (Pickford, et al., 1969; Friedel & Gillott, 1976), Diptera (Riemann & Thorson, 1969), and Lepidoptera (Benz, 1969; Yamaoka & Hirao, 1976). The possibility that males may affect the rate of oviposition as well as contribute nutrients for egg production has implications concerning the structure and evolution of insect mating systems.

Most investigations of spermatophore utilization have focused on the use of amino acids (Goss, 1977; Boggs & Gilbert, 1979; Boggs, 1981; Boggs

& Watt, 1981) or protein (Friedel & Gillott, 1977) by the female. To date, however, only Gerber et al. (1971) have presented data on the biochemical composition of spermatophores and the absorption of nonprotein components by the female. Their histochemical study of a meloid beetle spermatophore indicated presence of polysaccharides, proteins, phospholipids, and neutral lipids. In this study the spermatophore composition of *Colias philodice* and *C. eurytheme* spermatophores and temporal changes in their composition are described. In addition, a preliminary analysis of the contents of the appendix bursa, an organ associated with the bursa copulatrix (the receptacle for the spermatophore) (Figure 1), is presented.

Materials and Methods

Collection and Dissection of Butterflies

Colias philodice and *C. eurytheme* females were collected from May to October, 1979, in alfalfa fields at the Arizona State University Field Laboratory, Tempe, Arizona. From 28 August to 5 September 1979 *C. philodice* and *C. eurytheme* were sufficiently abundant to allow field collection of mating pairs. Copulating pairs were collected and transported in small vials to the laboratory within two hours of capture. The two species produce viable hybrids (Grula & Taylor, 1980a) and so are treated as one group here.

All spermatophores and appendix bursae were removed from females by dissection under water just prior to analysis. The duct between the bursa copulatrix and the appendix bursa was ligated with 6/0 silk. The appendix bursa was then separated from the bursa copulatrix (which contained the spermatophore) and both organs were placed in glass vials.

Spermatophores and appendix bursae from free flying, field collected females were divided into three classes using a qualitative technique

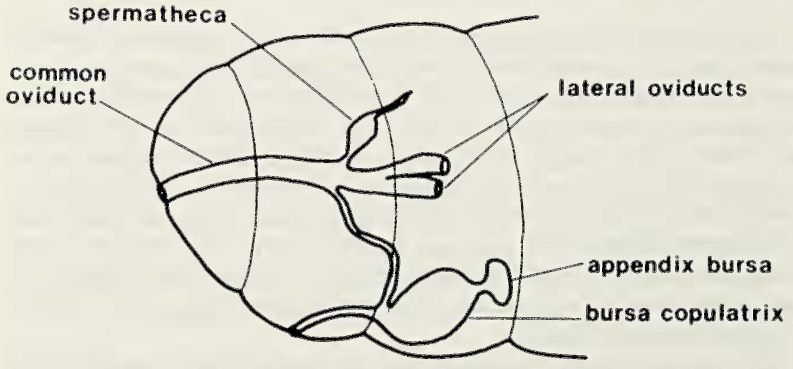

Fig. 1. Diagramatic view of the Lepidopteran female reproductive tract.

based on variation in size and shape of the structures and representing changes with time since deposition (Rutowski, 1981). These classes are: condition 1 — round and full; condition 2 — partially deflated; and condition 3 — almost completely deflated. Spermatophores and appendix bursae from field collected mating pairs are classified as newly mated (NM). These spermatophores are visually similar to condition 1 spermatophores.

Wet and Dry Mass

Single spermatophores and appendix bursae were placed in tared vials and wet mass determined to 0.01 mg. Samples were dried to a constant mass at 80°C.

Protein Analysis

Protein contents of both spermatophores and appendix bursae were analyzed using the BioRad method (Bradford, 1976). This colorphotometric technique enables quantification of protein but is insensitive to other forms of nitrogen. Single spermatophores were macerated and then digested for approximately 2 hr at room temperature in 2 ml in 1N NaOH. One tenth ml aliquots were transferred to sterile borosilicate culture tubes and 5 ml of diluted BioRad concentrate (5:1 with distilled water) added. Samples were mixed with a vortexer for 30 sec and allowed to react for a minimum of 5 min, after which the concentration of protein was estimated with a Bausch and Lomb Spectronic 20 at 595 nm using a bovine serum albumin standard curve. Three samples were analyzed for each spermatophore and the absorption values averaged.

Lipid Analysis

A. Extraction and Separation

Spermatophores were pooled by class (for pool sizes see Fig. 3) and allowed to stand for one hr in 5 ml of chloroform/methanol (2:1) at room temperature. The lipid extract was subjected to a complete Folch wash (Ways and Hanahan, 1964), evaporated to dryness under nitrogen, and weighed to 0.01 mg.

Total lipid samples were separated into three classes (hydrocarbons, nonhydrocarbons, and phospholipids) by silicic acid column chromatography (BioSil A) (Jackson, et. al., 1974). Following separation, each fraction was weighed to 0.01 mg.

B. Hydrocarbon Analysis

Argentation TLC, developed in hexane:diethyl ether:acetic acid (90:10:1 by volume), was used to check for unsaturation of the hydrocarbon fraction (Jackson, et al., 1974). The hydrocarbon fraction was analyzed by flame ionization detection gas chromatography using 183 x 0.32 cm glass columns packed with 3% OV — 101 on 100/120 Gas Chrom

Q; oven temperature was programmed for 220° to 300°C at 2°C/min. Peaks were identified by comparison to retention times of known standards and quantitated by electronic integration.

C. Nonhydrocarbon Analysis

Nonhydrocarbon fractions were analyzed by spotting a 1 ul sample on 250 um Silica Gel G plates (BioRad Laboratories) and developed in hexane:diethyl ether:formic acid (80:20:2 by volume) to separate major classes of polar lipids. Plates were charred and bands identified by comparison to known standards.

Results

Gravimetric Analysis of Male Imparted Secretions

Mean wet masses of newly implanted spermatophores and appendix bursae contents were 3.8 ± 1.9 mg and 0.88 ± 0.49 mg respectively, yielding a total mass transferred during mating of 4.7 mg (Fig. 1). Though these masses include the walls of the bursa copulatrix and appendix bursa it was found (by weighing both organs removed from virgin females) that the mass of the organs was negligible (less than 0.01 mg; N = 10). The mean body mass of field collected *C. philodice* and *C. eurytheme* males was 70.4 ± 15.4 mg (N = 35). Therefore, the mass of a new spermatophore plus appendix bursa contents represents 6-7 percent of a male's body mass.

Dry mass for newly implanted spermatophores and appendix bursa contents averaged 2.0 ± 1.1 and 0.35 ± 0.24 mg, respectively (Fig. 1). These masses declined significantly as spermatophore condition went from NM to 2 (Fig. 1) (Scheffe's test, $p < .05$).

Protein Analysis of Male Imparted Secretions

Spermatophore and appendix bursae contents from newly mated females contain 0.20 ± 0.12 and 0.088 ± 0.05 mg of protein, respectively (Fig. 2). As a percent of dry mass, this represents 10 percent from spermatophores and 23 percent from appendix bursae contents. Protein content in both spermatophores and appendix bursae declines as the organs deflate from condition 1 to 2 (Scheffe's test, $p < .05$) and nears zero in condition 3 appendix bursae (Fig. 2).

Lipid analysis of Male Imparted Secretions

A. Gravimetric Analysis

Total lipid contained in condition 1 spermatophores was 0.17 mg (Fig. 3), declining as spermatophore condition changed from condition 1 to 2 (Scheffe's test, $p < .05$) and then remained unchanged from condition 2 to condition 3 (Fig. 3). Separation of the total lipid fraction showed that hydrocarbon, nonhydrocarbon and phospholipid fractions represented, respectively, 47, 41, and 12 percent of the total lipid content of condition 1

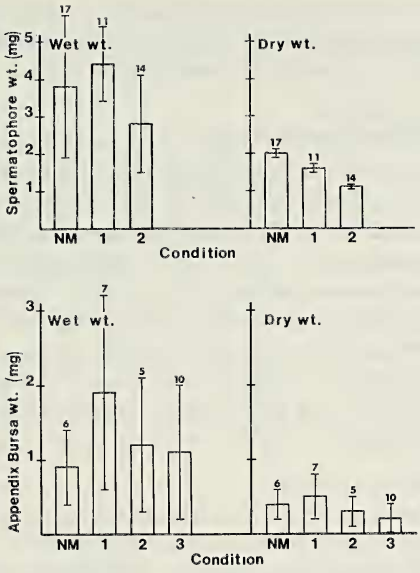

Fig. 2. Wet and dry masses of spermatophores and appendix bursae contents from *C. philodice* and *C. eurytheme* females. Numbers above each bar represent sample size. Brackets indicate one standard deviation. NM = newly mated. ND = no data available.

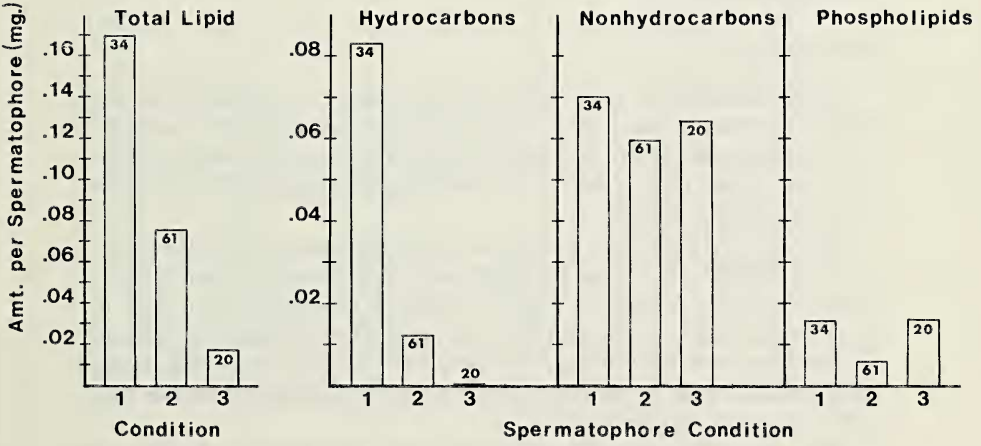

Fig. 3. The amount of protein per spermatophore (unhatched bars) and appendix bursae contents (hatched bars) from *C. philodice* and *C. eurytheme* females. Numbers above each bar represent sample size. Brackets indicate one standard deviation. NM = newly mated. ND = no data available.

spermatophores (Fig. 3). Only the hydrocarbon fraction declined as spermatophores deflated, while the weight of the nonhydrocarbon and phospholipid fractions remained constant (Fig. 3).

B. Hydrocarbon Analysis

Argentation TLC indicated that the hydrocarbons were completely saturated. Gas chromatographic analysis (GLC) of the total hydrocarbon fraction indicated the presence of over 25 components containing from 19 to 30 carbon atoms (Table 1). Normal alkanes represent 93 percent of the total hydrocarbon extracted, with chain lengths ranging between 25 and 29 accounting for 51 percent of the total (Table 1). There appear to be no significant differences in hydrocarbon composition between conditions 1 and 2 spermatophores. Due to lack of material, only one GLC separation was possible with condition 3 spermatophores and though no averaging of GLC data was possible, this single trace indicates no compositional differences between condition 3 and conditions 1 and 2 spermatophores.

C. Nonhydrocarbon Analysis

Visual analysis of charred TLC plates of the nonhydrocarbon fraction indicates that this fraction of spermatophore lipid contains sterols, diglycerides, triglycerides, and free fatty acids in nearly equal amounts with traces of alcohols and sterol esters as well. Because recoverable amounts were small, no attempt was made to quantify these components. While such visual analysis is admittedly crude, it indicates no change in composition of this fraction as spermatophores deflate from condition 1 to condition 3.

Discussion

During copulation a male *Colias philodice* and *C. eurytheme* fills the female's appendix bursa with a white, protein-rich substance and constructs within the bursa copulatrix a spermatophore containing both proteins and lipids. This deposition represents approximately 6 to 7 percent of the male's body mass.

There are two ways male butterflies could increase the number of offspring produced per mating by supplying material via a spermatophore. Males could supply chemicals that increase the rate of egg production, or males could supply nutrients that decrease the time required by females for foraging, and thus increase available oviposition time. This study indicates that male *C. philodice* and *C. eurytheme* supply materials that could operate in both ways.

Protein Content of Male Imparted Secretions

Protein is an important component of spermatophores representing approximately 10 and 23 percent, respectively, of spermatophore and appendix bursa dry masses, and 12 percent of total male secretion. Pro-

Table 1. Identification and percentage composition of hydrocarbons from condition 1 and condition 2 spermatophores of *Colias philodice* and *C. eurytheme* butterflies.

GLC Peak No.	ECL	Percentage Composition		Alkane
		Condition 1	Condition 2	
19	19	trace*	1.7	n-Nonadecane
19-B	19.6	—	trace	unknown
20	20	trace	.8	n-Eicosane
21	21	1.6	1.7	n-Heneicosane
22	22	3.6	2.6	n-Docosane
23	23	6.4	5.2	n-Tricosane
24	24	8.1	6.5	n-Tetracosane
25	25	10.1	8.6	n-Pentacosane
26	26	9.7	8.6	n-Hexacosane
26-B	26.6	trace	trace	unknown
27	27	10.0	9.2	n-Heptacosane
27-B	27.6	trace	—	unknown
28	28	12.8	12.8	n-Octacosane
28-B	28.4	—	.7	unknown
29	29	9.1	9.5	n-Nonacosane
30	30	5.5	6.4	n-Triacontane
30-B	30.6	—	.6	unknown
31	31	5.5	5.9	n-Hentriacontane
32	32	3.1	3.6	n-Dotriacontane
33	33	1.5	2.6	n-Tritriacontane
34	34	1.6	1.0	n-Tritetracontane
35	35	1.6	1.0	n-Tripentacontane
35-B	35.2	1.1	.7	unknown
36	36	1.1	trace	n-Trihexacontane
36-A	36.2	trace	—	unknown
36-B	36.6	.8	1.3	unknown
37	37	trace	—	n-Triheptacontane
38	38	2.1	1.6	n-Triheptacontane
		95.2	92.2	

* less than 0.5%
ECL = equivalent chain lengths

teins are important constituents of insect eggs (Clayton & Edwards, 1961) and, while some protein components (amino acids) are available to adult butterflies from dietary sources (Baker & Baker, 1973), males, by supplying protein or its components could increase time available for oviposition by decreasing the amount of time required by the female for foraging.

Rutowski (1978) has shown time to be important to ovipositing female in *C. philodice* and *C. eurytheme*.

Lipid Content of Male Imparted Secretions

Total lipid recovered from spermatophores represents approximately 9 percent of dry mass, about the same amount as recovered spermatophore protein (10 percent). The lipid fraction is composed of nearly equal fractions of hydrocarbons and nonhydrocarbons (cholesterol, diglycerides, triglycerides, and free fatty acids) and small amounts of phospholipids.

The hydrocarbon fraction, either intact or following degradation, leaves the spermatophore while other fractions persist. Hydrocarbons are used as waterproofing agents by many insects (see Jackson & Baker, 1970 for review) and may be used by female insects to waterproof eggs, being incorporated during chorionation. Synthesis of hydrocarbons by insects is mediated by ecdysone (Arnold & Regnier, 1975), which is at low levels at eclosion (Riddiford & Truman, 1978). Since copulation immediately follows female eclosion males may allow females to oviposit sooner by supplying hydrocarbons. It is interesting to note that 51 percent of the hydrocarbon fraction is of C_{25}-C_{29} chainlengths. The male pheromone (received by the female during courtship) contains C_{23}, C_{25}, C_{27} and C_{29} hydrocarbons (Grula & Taylor, 1980b). By assessing male pheromone production during courtship females may be able to determine a courting male's ability to produce a spermatophore. This could explain Rutowski's observation (1979) that previously mated males were much less persistent in courtship.

The nonhydrocarbon fraction consists of approximately equal amounts of sterols, diglycerides, triglycerides and free fatty acids. These components are the major nonhydrocarbon components found in insect eggs (Svoboda, et al., 1966). It is reasonable to suggest that male nutrient investments of this nature may substantially decrease the time required for female foraging. Further, since sterols cannot be synthesized by insects (Clayton & Edwards, 1961) availability of this nutrient may directly limit egg production.

It is obvious from the discussion above that many questions remain unanswered about spermatophores and their functions as paternal investment. It is clear however, that spermatophores can no longer be viewed as just sperm or a single resource. Spermatophores are a complex of chemicals, any or all of which could potentially increase the number of offspring resulting from a mating.

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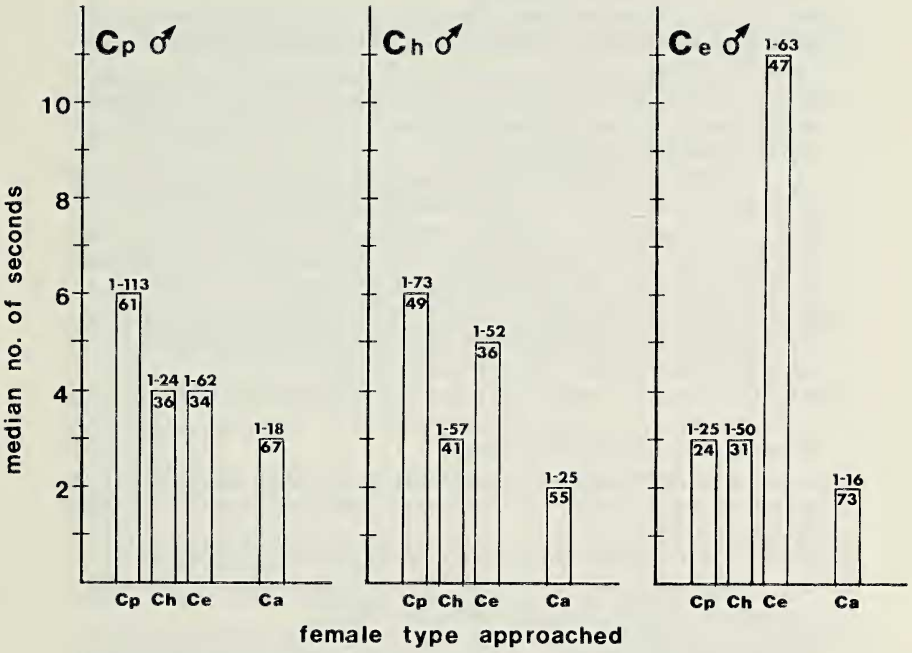

Fig. 4. Total lipid and the 3 major classes of lipids extracted from spermatophores of *C. philodice* and *C. eurytheme*. Numbers within each bar represent the number of spermatophores within each pool.

Literature Cited

ARNOLD, M. T. & F. E. REGNIER, 1975. Stimulation of hydrocarbon biosynthesis by ecdysterone in the fleshfly *Sarcophaga bullata*. Journal of Insect Physiology 21:151-1586.

BAKER, H. G. & I. BAKER, 1973. Amino acids in nectar and their evolutionary significance. Nature 241:543-545.

BENZ, G., 1969. Influence of mating, insemination, and other factors on oogenesis and oviposition in the moth *Zieraphera diniana*. Journal of Insect Physiology 15:55-71.

BOGGS, C. L., 1981. Selection pressures affecting male nutrient investment at mating in heliconine butterflies. Evolution 35:932-940.

BOGGS, C. L. & L. E. GILBERT, 1979. Male contribution to egg production in butterflies: evidence for transfer of nutrients at mating. Science 206:83-84.

BOGGS, C. L. & W. WATT, 1981. Population structure of pierid butterflies. IV. Genetic and physiological investment of offspring by male *Colias*. Oecologia 50:320-324.

BRADFORD, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding.

Analytical Biochemistry 72:248-254.

CLAYTON, R. B. & A. M. EDWARDS, 1961. The essential cholesterol requirement of the roach *Eurycotis floridana*. Biochemical and Biophysical Research Communications 6:281-284.

FRIEDEL, T. & C. GILLOTT, 1976. Male accessory gland substance of *Melanoplus sanguinipes*: an oviposition stimulant under the control of the corpus allatum. Joural of Insect Physiology 22:489-495.

_____, 1977. Contribution of male-produced proteins to vitellogenesis in *Melanoplus sanguinipes*. Journal of Insect Physiology 23:145-151.

GERBER, G. H., N. S. CHURCH & J. G. KEMPEL, 1971. The structure, histochemical, fate, and functions of the spermatophore of *Lytta nuttalli* Say (Coleoptera: Meloidae). Canadian Journal of Zoology 49:1595-1610.

GOSS, G. J., 1977. The interaction between moths and pyrrolizidine alkaloid-containing plants including nutrient transfer via the spermatophore in *Lymire edwardsii* (Ctenuchidae). Univ. of Miami, Ph.D.

GRULA, J. W. & O. R. TAYLOR, 1980a. Some characteristics of hybrids derived from the sulfur butterflies, *C. eurytheme* and *C. philodice*: Phenotypic effects of the X-chromosome. Evolution 34:673-687.

_____, 1980b. Aphrodisiac pheromones of the sulfur butterflies *Colias eurytheme* and *C. philodice* (Lepidoptera, Pieridae). Journal of Chemical Ecology, 241-256.

JACKSON, L. L. & G. L. BAKER, 1970. Cuticular lipids of insects. Lipids 5:1-8.

JACKSON, L. L., M. T. ARNOLD & F. E. REGNIER, 1974. Cuticular lipids of adult fleshflies, *Sarcophaga bullata*. Insect Biochemistry 3:369-379.

PICKFORD, R., A. B. EWEN & C. GILLOT, 1969. Male accessory gland substance: an egg-laying stimulant in *Melanoplus sanguinipes* (Orthoptera: Acrididae) Canadian Journal of Zoology 47:1199-1203.

RIDDIFORD, L. M. & J. M. TRUMAN, 1978. Biochemistry of insect hormones and growth regulators. *In* Biochemistry of Insects ed. M. Rockstein, Academic Press.

RIEMANN, J. G. & B. J. THORSON, 1969. Effect of male accessory material on oviposition and mating by female house flies. Annals of the Entomological Society of America 62:828-834.

RUTOWSKI, R. L., 1978. Form and function of ascending flights in *Colias* butterflies. Behavioral Ecology & Sociobiology 3:163-172.

_____, 1979. The butterfly as an honest salesman. Animal Behavior 27:1269-1270.

_____, 1980. Courtship solicitation by females of the checkered white butterfly, *Pieris protodice*. Behavioral Ecology & Sociobiology 7:113-117.

RUTOWSKI, R. L., C. E. LONG, L. D. MARSHALL & R. S. VETTER, 1981. Courtship solicitation by *Colias* females (Lepidoptera: Pieridae). amer. Midl. Nat. 105:334-340.

SVOBODA, J. A., J. H. PETTER & G. L. BAKER, 1966. On the lipids of the egg of the grasshopper, *Aulocara elliotti*. Journal of Insect Physiology 12:1549-1565.

THORNHILL, A. R., 1976. Sexual selection and paternal investment in insects. American Naturalist 110:153-163.

WAYS, P. & D. J. HANAHAN, 1964. Characterization and quantification of red cell lipids in normal man. Journal of Lipid Research 5:318-328.

YAMAOKA, K. & T. HIRAO, 1977. Stimulation of virginal oviposition by male factor and its effect on spontaneous nervous activity in *Bombyx mori*. Journal of Insect Physiology 23:57-63.